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Award Number: DAMD17-01-1-0255

TITLE: Examination of the Contribution of VEGF to the Metastatic  
Dissemination of c-Myc Overexpressing Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Michael Johnson, M.D.

CONTRACTING ORGANIZATION: Georgetown University  
Washington, DC 20007

REPORT DATE: July 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20050105 016

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> July 2004	<b>3. REPORT TYPE AND DATES COVERED</b> Final (1 Jul 2001 - 30 Jun 2004)	
<b>4. TITLE AND SUBTITLE</b> Examination of the Contribution of VEGF to the Metastatic Dissemination of c-Myc Overexpressing Breast Cancer Cells			<b>5. FUNDING NUMBERS</b> DAMD17-01-1-0255	
<b>6. AUTHOR(S)</b> Michael Johnson, M.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Georgetown University Washington, DC 20007  <i>E-Mail:</i> Johnson@georgetown.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b>  Metastasis to the lung is a common occurrence accounting for approximately 60-70% of metastasis. We have developed a transgenic model of lung metastasis using a MMTV-c-myc/MMTV-VEGF bitransgenic mouse system. This system and fluorescent variants thereof have been used to examine the importance of VEGF to the metastatic behavior of these cells. We have shown that, surprisingly, the MMTV-c-myc model in fact exhibits much more metastatic potential than previously appreciated, but that c-myc/VEGF cells produce a dramatic stromal reaction in the lung of mice which results in considerable morbidity. The use of EGFP expressing mice as hosts for red-fluorescent tumor cells has greatly facilitated our ability to examine the processes of extravasation and local tumor cell spread.				
<b>14. SUBJECT TERMS</b> Vascular endothelial growth factor, c-myc, intravasation, estravasation metastasis				<b>15. NUMBER OF PAGES</b> 22
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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## **Introduction:**

Angiogenesis is a process that has been shown to be of critical importance to mammary tumor development growth and dissemination. There is a dearth of models for the study of these tightly linked processes – angiogenesis and metastasis, since none of the available human breast cancer cell lines are metastatic in the nude mouse and many of the available mouse models are driven by oncogenes not normally overexpressed in human tumors.

The oncogene c-myc is highly relevant to human cancer, however, being amplified or overexpressed in a significant proportion of human breast tumors. Mice that carry a transgene in which the MMTV (Mouse Mammary Tumor Virus) promoter directs the expression of c-myc to the mammary glands, develop mammary tumors after multiple pregnancies. However, these tumors are relatively poorly vascularized and typically only form rare, small metastases in the lungs of the mice.

We hypothesized that increasing the expression of the angiogenic growth factor VEGF (Vascular Endothelial Growth Factor) in these tumors would result in increased vascularization of the tumors and increased metastatic potential. In order to achieve this goal, we crossed the MMTV-c-myc mice with another transgenic mouse strain which carry a MMTV driven VEGF expression cassette. The frequency and latency of tumor formation in these mice is unchanged, but as hypothesized, the tumors are much more vascular and metastasize more frequently forming larger secondary tumors.

VEGF-A is highly relevant to human tumors. Many human breast cancers express significant levels of VEGF and so the model we have created accurately mimics at least one variety of invasive and metastatic mammary tumor. It is reassuring that by standard histological criteria the tumors that occur in these mice indeed look very similar to human breast cancers and are quite distinct from the tumors that naturally occur spontaneously within the mammary glands of mice, or those that are seen in carcinogen induced models of mammary carcinogenesis.

## **Body:**

### **Summary of research accomplished:**

As will be outlined below in this final report, we have essentially completed all of the goals of the proposal as originally outlined and have also completed some additional studies which are the basis of ongoing studies. Some of the data generated are rather less interesting than we had originally thought that they might be, however, the reason that this is so represents an interesting, novel and potentially important finding in itself. The proposal consisted of two aims: 1) to evaluate the contribution of VEGF to the extravasation of c-myc and c-myc/VEGF tumors, and 2) to evaluate the contribution of VEGF to intravasation from the primary tumor. Aim 1 was to be achieved by a detailed analysis of the biology of metastatic deposits of c-myc or c-myc/VEGF cells generated by tail-vein injection, whereas Aim 2 was to examine in detail the biology of the primary

tumors and the dynamics of tumor cell release by the primary tumors. The idea was that the study would capitalize on the difference between the c-myc tumors and the c-myc/VEGF tumors to get a handle on the effect that the VEGF was having. As described in the previous reports, we ran into some technical difficulties during the course of the study relating to our animal colonies. Fecundity issues and other problems significantly slowed the accrual of sufficient specimens for the study and an additional year was requested and granted in the form of a no-cost extension of the project. The extra time allowed us to generate the animals required and to age them such that the necessary tumors developed. Whilst this was going on we were developing several cell lines from tumors from c-myc and c-myc/VEGF mice using a novel, highly effective and simple method which along with a characterization of several of the cell lines generated, we have now published (1). These lines were then transfected with expression plasmids that direct either the expression of the green fluorescent protein EGFP, or the red fluorescent protein DSRRed-2 for use in Aim 2 of the project.

Using red and green fluorescently labeled c-myc and c-myc/VEGF tumor derived cell lines we initiated studies to investigate the *in vivo*, growth, invasiveness and metastatic properties of these cells. Both transgenic mouse lines (c-myc and VEGF) being on an FVB background, the resultant cell lines are syngeneic with regular FVB mice and so we had the luxury of being able to use FVB mice, rather than an immunocompromised model as would have been the case with xenograft studies. This has the obvious advantage that the studies are conducted in the presence of an intact immune system, which as described below is, we believe, important. As these studies progressed, it became apparent that there was much less difference in terms of metastatic ability between the c-myc and the c-myc/VEGF cells than we were expecting. Whilst the c-myc/VEGF cells were indeed able to metastasize very rapidly as and quickly killed the mice due to pulmonary problems, the c-myc cells also were able to metastasize quite efficiently, though the resultant metastatic deposits were less likely to rapidly kill the animals. (It should be noted here for the record that death of the animals was never an end-point. Mice were monitored closely and sacrificed as soon as it became apparent that they were in any distress). This finding was repeated with several cell lines and we consistently found that the c-myc cells had much greater metastatic potential than we had expected. This finding prompted us to wonder if this was the result of some sort of selective pressure in the generation of the cell lines, some sort of tissue culture artifact, or a property of the parent tumors that we had not appreciated. Previous published work, including our own, has failed to report significant incidence of metastatic disease in MMTV-c-myc mice with mammary tumors (2, 3, 4). However, none of these studies was specifically looking at metastasis as an end point, although we had always looked for evidence of metastasis in all our work with the mice. Nevertheless, we initiated a detailed re-analysis of the samples we had generated and were generating for this study. With the assistance of a veterinary pathologist recently recruited to the institution, we altered the way we were processing the lung tissues from the mice to facilitate the detection of occult metastasis. The result of this work was the finding that in fact, approximately 30% of c-myc mice with tumors had significant metastatic deposits in their lungs at the time of sacrifice. In the previous annual reports that we have submitted in connection with this study, we have reported the paper "VEGF-dependent mammary

tumor metastasis in a novel bi-transgenic model. Nobel et al", as being in preparation. We must again report this paper to be "in preparation" at this time, since this new understanding of the model has necessitated our re-writing the paper. As mentioned above, we have gone back and re-analyzed archival samples from our earliest work with the MMTV-c-myc model and have found that indeed this level of metastasis was seen in our original studies and missed since they were not focused on metastasis so much as tumor incidence and characteristics. This strongly suggests that this has been an unappreciated feature of this model all along. This is an important finding since it speaks to the additional relevance of an already important mouse model of human cancer. The paper under preparation will describe this feature of the model.

Another important implication of this finding is it enhances the relevance of our observations with the transplanted cell lines. We see roughly equivalent rates of metastasis from tumors that are derived from transplanted myc, or myc/VEGF cells as we see in transgenic animals that have developed tumors spontaneously. Thus, neither the trauma of the initial introduction of the tumor cells, nor changes in those cells during their isolation, propagation in vitro, or transduction with fluorescent proteins seems to have significantly altered their properties.

In summary, approximately 30% of c-myc tumors and 60% of c-myc/VEGF tumors result in metastasis to the lungs. While rates appear somewhat higher for the transplanted cells – ultimately almost 100% of mice injected with c-myc/VEGF cells will develop metastatic deposits of the disease, this difference is most likely, we believe, a function of the timing and rapidity of growth. The transplanted tumors grow much more rapidly than the spontaneous tumors, they undergo metastasis earlier, and the metastatic deposits are more aggressive. The bi-transgenic mice develop tumors relatively late in their life, which grow relatively slowly and which metastasize later in the course of the disease. Under the terms of our animal protocol we sacrifice our transgenic mice at one year of life and we believe that this artificial cut off in the natural course of the disease results in a lower incidence of metastasis than we would see if the mice were allowed to age further. Thus, the transplant system is apparently an accelerated model of the natural outcome of the disease in the transgenic animals.

The principal goal of Aim 1 was to conduct a detailed immunohistochemical analysis of metastatic deposits in the lungs of mice injected with c-myc vs c-myc/VEGF tumors to look at the various receptors for VEGF and for markers of apoptosis and proliferation to address the issue of how the expression of VEGF makes these deposits more aggressive. We have confirmed our original findings that the c-myc/VEGF tumors are both more proliferative and less apoptotic than the c-myc tumors, though these effects are less pronounced than one would expect if this were the principal reason for the differences in the biology seen. Studies to look at the levels of the various VEGF receptors have been somewhat hampered by a dearth of good antibodies against these proteins that work well in these contexts and so these studies are still underway. However, in order to circumvent some of these difficulties and to come at the problem from a different angle we have made use of some additional strategies not originally proposed. The availability of expert veterinary pathological support and a new laser capture micro-dissection system



has made possible the isolation of mRNA from these lesions. This in combination with the powerful techniques of expression array analysis and truly quantitative PCR measurement of expression levels have allowed us to start analyzing the genes of interest and to trawl for other genes that we had not imagined might be part of the biology we have seen. These studies are ongoing. We have also conducted a series of *in vitro* studies to probe the signaling of c-myc versus c-myc/VEGF cells with the endothelial components of the tumor. The goal of these studies has been to define the key molecules that are being stimulated to generate the enhanced vasculature seen in the c-myc/VEGF metastases. We have shown that this communication is quite degenerate with several possible receptors being engaged. Inhibitor studies have suggested however that flk-1 is the most important molecule in this system – both *in vitro* and *in vivo*. Overall, from the data we have so far from all of these approaches, there does not seem, in fact to be a dramatic difference in the level or disposition of any of the proteins we had originally thought might be the key to the different behavior of the cells in this system. However, it has become evident that one feature of the system is very important.

In the first of the annual reports that we submitted in connection with this proposal, one of the technical challenges we mentioned that we had to come to grips with was the mysterious morphology of some of the tumors and metastatic deposits derived from the cell lines we were working with. Indeed, so strange was the morphology that the pathologist we were working with at that time, was convinced that we had misidentified the cells and that they must have been isolated from a non-mammary tumor. This lead us to re-derive the cell lines and was part of the reason we needed the no-cost extension. We are now convinced that the original cells were in fact fine and that the effects we were seeing are not an artifact, but a critical and important aspect of the biology of the system. It seems that some factor on the surface of, or elaborated from the c-myc/VEGF cells results in sometimes quite dramatic alterations in the morphology of the tissue surrounding the tumor cells. Indeed, it seems that this is a large part of the morbidity that is produced by the metastatic deposits in mice with these tumors. It is not clear that this is in fact a direct role of the VEGF, since experiments in which we have tried to recapitulate the phenotype by transfecting c-myc cells with a VEGF expression vector have not produced these effects. Indeed it was not clear for some time if the aberrant cells were in fact cells from the lung co-opted by the tumor cells, or tumor cells that had altered their morphology.

To address the issue of the provenance of these cells, we initiated a series of studies which have proven to be extremely profitable and which has opened a whole new avenue of research for this project. As described above, we have labeled the c-myc/VEGF cells with DSRed-2, which is a naturally red-fluorescent protein. These cells are syngeneic with inbred FVB mice and so can be grown as grafts on these animals. However, using conventional microscopy, our ability to appreciate the interplay of the tumor cells with the host tissues is limited. We, therefore, obtained a transgenic strain originally created by Andres Nagy in which the green fluorescent protein EGFP is expressed off a CMV enhanced chicken actin promoter. Thus essentially every cell of these mice contains some level of the EGFP. These mice have been back-crossed onto the FVB line and so we can grow our red-fluorescent cells in green-fluorescent mice. This has dramatically

enhanced our ability to study the interplay of the tumor cells with the mouse cells and the most basic level allows us to easily distinguish one from the other. From these studies it has become apparent that the cells that exhibit the morphologies that gave our original pathologist so much difficulty are indeed a stromal reaction to the tumor cells – presumably caused by some factor elaborated by the cells. The exact nature of the cells involved is still somewhat of a mystery and we are conducting studies to look at much earlier time points in the hope that we will be able to accurately characterize the key players. So far we know that the cells involved include both structural components of the lung tissue its self and cells of some hematopoietic lineage. Again, we don't believe that the factor being elaborated by the cells is VEGF itself or is directly related to VEGF expression, since simply transfecting the c-myc cells with a VEGF expression vector does not recapitulate the effect. We are hopeful that the array based experiments we are conducting will shed some light on this intriguing phenomenon.

As mentioned above, the use of the fluorescent mice in combination with the fluorescent cells has also greatly facilitated the work we have done on Aim-2. The goal of this aim was to examine the contribution of VEGF to tumor intravasation. In addition to the FACS based studies that were proposed in the original application, we have also been able to use fluorescence based microscopic approaches to directly visualize the movement and intravasation of tumor cells. Somewhat disappointingly we have not found as much difference between the c-myc and the c-myc/VEGF cells as we had anticipated in the original application. With what we have learned about the c-myc model, this is not, in hind site particularly surprising. We have after all shown, as described above, that the c-myc tumors can indeed metastasize at a much higher rate than was previously appreciated. This is consistent with our evidence that majority of the difference seen in terms of outcome in these models is what happens at the site of metastasis rather than at the primary tumor. However, the methods we have developed in this study have allowed us to examine the process of tumor cell intravasation in an entirely novel and fruitful way.

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- 1) Pei, X.P., Noble, M.S., Davoli, M.A., Rosfjord, E., Tilli, M.T., Furth, P.A., Russell, R., Johnson, M.D. and Dickson, R.B. (2004) Explant-cell culture of primary mammary tumors from MMTV – c-Myc transgenic mice. *In Vitro Cell Dev. Biol. Anim* 40(1):14-21.
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#### Key Research Accomplishments:

- 1) We have discovered that the MMTV-c-Myc mouse model is much more metastatic than previously believed.
- 2) We have developed an facile, reproducible, widely applicable method for the generation of cell lines from tumors from transgenic mice.
- 3) We have demonstrated the importance of early parity to tumor development in the MMTV-c-myc model
- 4) We have developed novel and extremely powerful methods for studying the process of intravasation using fluorescence techniques
- 5) We have show that c-myc/VEGF derived tumor cells have a dramatic ability to co-opt the stromal components of the mouse lung and immune system to

#### Reportable Outcomes:

Jamerson, M.H., Johnson, M.D., Furth, P.A. and Dickson R.B. (2003) Early parity significantly elevates mammary tumor incidence in MMTV-c-myc transgenic mice. *Transgenic Res.* 12:747-50

Pei, X.P., Noble, M.S., Davoli, M.A., Rosfjord, E., Tilli, M.T., Furth, P.A., Russell, R., Johnson, M.D. and Dickson, R.B. (2004) Explant-cell culture of primary mammary tumors from MMTV – c-Myc transgenic mice. *In Vitro Cell Dev. Biol. Anim* 40(1):14-21.

VEGEF-dependent mammary tumor metastasis in a novel bitransgenic model. Nobel et al, in preparation.

#### Conclusions:

This project set out to examine the contribution of VEGF the metastatic dissemination of c-myc overexpressing breast cancer cells. This goal was based on the premise that MMTV-myc tumors were much less metastatic than MMTV-myc/VEGF tumors. However, as we have described, MMTV-myc tumors are in fact quite metastatic, and the key difference between these models relates to the behavior of the metastatic deposits that result. We have not seen dramatic differences in the ability of the c-myc or the c-myc/VEGF cells to extravasate nor their ability to survive in the circulation, or to establish metastatic deposits in the lung. We have, however, seen a dramatic difference in terms of the biology of the metastatic deposits that result. However, it is far from clear that these effects relate directly to the expression of VEGF since attempts to re-capitulate

the phenotype by simple transfection of c-myc cells with VEGF have not been successful. While it is somewhat disappointing that the original objectives of the project have not proven to yield particularly interesting data, the proposal has yealded important findings. We have shown that the MMTV-c-myc model is if anything more valuable that had previously been appreciated and we will shortly publish our paper describing these findings and the full characterization of the MMTV-c-myc model. This proposal has supported the development of a powerful new method of generating cell lines from tumors from transgenic mice which we have recently published. This method, now widely used by several groups in this institution and elsewhere, allows considerable additional value to be gained from transgenic animal models. Perhaps most importantly, however, the study has allowed us to develop a powerful new method for studying the metastatic process in live tissue by fluorescence which we believe will yield extremely valuable data in the years to come.

Abbreviations:

EGFP	Enhanced Green Fluorescent Protein
GFP	Green Fluorescent Protein
IHC	Immunohistochemistry
MMTV	Mouse Mammary Tumor Virus
RFP	Red Fluorescent Protein
VEGF	Vascular Endothelial Growth Factor

Appendices:

Persons receiving pay from the research effort:

Michael D. Johnson, PhD  
Hong Zuo, MD

Copies of published papers:



Short communication

## Early parity significantly elevates mammary tumor incidence in MMTV-*c-myc* transgenic mice

M. Hunter Jamerson, Michael D. Johnson, Priscilla A. Furth & Robert B. Dickson\*

Department of Oncology and Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington, DC 20057, USA

Received 25 May 2003; revised 21 August 2003; accepted 28 August 2003

**Key words:** *c-myc*, mammary gland, mouse mammary tumor virus, pregnancy, transgenic mice, tumorigenesis

### Abstract

Female transgenic mice, in which a murine *c-myc* gene has been placed under the transcriptional control of the mouse mammary tumor virus long terminal repeat, are prone to developing mammary adenocarcinomas. Owing to the manner in which these mammary tumors develop, it is clear that exogenous expression of the *c-myc* transgene is necessary to but insufficient for murine mammary tumorigenesis. The genetic background of study mice has been shown to influence the phenotype induced by different transgenes; furthermore, mammary tumor initiation and progression induced by different transgenes has been shown to be susceptible to significant modification with alterations in and mixing of the genetic background of the study mice. We bred MMTV-*c-myc* transgenic mice onto a mixed genetic background that resulted in a very significant suppression of mammary tumor incidence for parous mice, bred continuously starting at 10 weeks of age. In this paper, we show that mammary tumor incidence is significantly elevated in these mixed background MMTV-*c-myc* transgenic mice when they are bred continuously, starting at 7 weeks of age. Early breeding of these mice did not influence mammary tumor multiplicity, latency, histopathology, or number of pregnancies at time of tumor development. These results are the first to demonstrate that breeding age influences mammary tumor incidence in MMTV-*c-myc* transgenic mice. They suggest that mammary gland susceptibility to tumorigenesis, resulting from the expression of *c-myc*, may vary with glandular development as is seen for chemical carcinogens.

### Introduction

The first transgenic mouse generated to evaluate the relevance of a cellular proto-oncogene to mammary development and tumorigenesis utilized the mouse mammary tumor virus long terminal repeat (MMTV-LTR) sequence to target the expression of *c-myc* to the mammary epithelium of virgin, pregnant and lactating mice (Donehower et al., 1980; Stewart et al., 1984; Leder et al., 1986). Female MMTV-*c-myc* mice develop mammary adenocarcinomas in the multiparous state ( $\approx 100\%$  penetrance with  $\geq 2$  pregnancies) more frequently and rapidly than in the virgin state ( $\approx 50\%$

with 7–14-month latency) (Leder et al., 1986). This disparity may result from pregnancy-related mammary epithelial cell (MEC) proliferation and survival, opposing the pro-apoptotic influence of deregulated *c-myc* expression, and from stimulation of transcriptional activity from the MMTV-LTR by the hormones of pregnancy and lactation (Donehower et al., 1981). Transgene-induced mammary tumorigenesis reflects the nature and context of the expressed transgene as well as the pathways activated and/or repressed by expression of the transgene (Cardiff, 1996; Cardiff et al., 2000; Desai et al., 2002). It is clear that transgene-dependent phenotypes, including transgene-induced mammary tumorigenesis, can be significantly altered by the murine genetic background on which these genetic alterations are studied (Lifsted et al., 1998;

\* Author for correspondence  
E-mail: dicksonr@georgetown.edu

Table 1. Comparison of mammary tumor characteristics for MMTV-*c-myc* transgenic mice bred at 7 weeks of age versus 10 weeks age

	Early parous		Parous	
	MMTV- <i>c-myc</i>	Non-transgenic	MMTV- <i>c-myc</i>	Non-transgenic
<i>n</i>	23	10	12	17
Tumor-bearing mice	20	0	2	0
Tumor incidence ± SEM	87% ± 7.2%	0%	16.7% ± 11.2%	0%
Tumor multiplicity ± SEM	2.4 ± 0.31	na	2.5 ± 0.50	na
Tumor latency (days)	164.3 ± 6.9	na	174 ± 9.0	na
Parity at time of tumor(s)	3.55	na	4.5	na

Rowse et al., 1998; Doetschmann, 1999; MacLeod & Jacks, 1999; Le Voyer et al., 2000).

As part of another study examining the potential for cooperation between *c-myc* and *bcl-x<sub>L</sub>* transgenes in mammary tumorigenesis, we bred MMTV-*c-myc* transgenic mice onto a mixed genetic background. In parous mice expressing only the *c-myc* transgene, we found that the mammary tumor incidence was very significantly decreased in mice on a mixed genetic background as compared to the inbred FVB genetic background. In the present paper, we report that the mammary tumor incidence in MMTV-*c-myc* transgenic mice on this mixed genetic background was significantly increased when female mice were bred at 7 weeks of age, as compared to at 10 weeks of age. This study is the first published to demonstrate changes in tumor incidence associated with altered timing of pregnancy in female transgenic mice.

## Materials, results and discussion

### *Generation and breeding of mixed genetic background MMTV-c-myc transgenic mice*

MMTV-*c-myc* transgenic mice, originally on the FVB inbred genetic background, were bred onto a mixed FVB × C57BL/6J × C3HeB/FeJLe genetic background and the presence or absence of the *c-myc* transgene was confirmed by PCR-based genotyping using MMTV-*c-myc* transgene-specific primers as previously reported (Amundadottir et al., 1995). Female MMTV-*c-myc* and non-transgenic study mice were aged 10 (parous) or 7 ('early parous') weeks and then co-housed with male mice and bred continu-

ously. As shown in Table 1, the parous breeding group was composed of 12 MMTV-*c-myc* transgenic and 17 non-transgenic female mice, while the early parous breeding group contained 23 MMTV-*c-myc* transgenic and 10 non-transgenic female mice. To prevent litters from overlapping, all surviving pups from each litter were weaned at post-partum day 20. All female study mice were palpated for tumors bi-weekly through 13 months of follow-up and all tumor-bearing mice were euthanized prior to the point where their tumor burden equaled 10% of their body weight. All mammary tumors were fixed in neutral-buffered formalin and embedded in paraffin by routine histopathological methods.

### *Mammary tumor characteristics in early parous bred versus parous bred, mixed genetic background MMTV-c-myc transgenic mice*

As shown in Table 1, mammary tumors were only observed in *c-myc* transgene-positive study mice and not in non-transgenic study mice. Breeding female MMTV-*c-myc* transgenic study mice at 7 weeks of age ('early parous') versus 10 weeks of age ('parous') resulted in a statistically significant ( $p < 0.001$ , ANOVA and Tukey post-hoc testing), five-fold elevation in mammary tumor incidence (87% for early parous and 16.7% for parous *c-myc* study mice). Breeding female MMTV-*c-myc* transgenic study mice at 7 weeks of age versus 10 weeks of age did not result in any statistically significant alterations in mammary tumor multiplicity ( $p = 1.000$ ), mammary tumor latency ( $p = 0.998$ ), or mean parity at time of mammary tumor development ( $p = 0.930$ ). Furthermore, no correlation was identified between breeding age and mammary

tumor histopathological character as both early parous and parous female MMTV-*c-myc* transgenic study mice developed a mixture of dense glandular and moderately dense cribriform glandular adenocarcinomas (data not shown). These glandular adenocarcinomas were typical of mammary tumors arising in MMTV-*c-myc* transgenic mice (Cardiff et al., 2000). Mammary gland whole-mounts from tumor-bearing study mice exhibited prominent and widespread hyperplastic alveolar nodular changes in areas without gross tumor involvement, whereas whole mounts taken from study mice that did not develop adenocarcinomas were without such hyperplastic alterations (data not shown).

### Conclusions and discussion

Early breeding of mixed strain, female MMTV-*c-myc* transgenic study mice significantly elevates mammary tumor incidence without altering other mammary tumor characteristics such as mammary tumor histopathology, latency, multiplicity and parity at time of mammary tumor development. Microscopic histopathological evidence suggests that exogenous expression of the *c-myc* transgene is responsible for transformation of the mammary gland in both early parous bred and parous-bred mice. Furthermore, mammary adenocarcinomas that developed in these two groups of mice were of similar histopathological character and were not different from mammary adenocarcinomas previously identified in other studies of MMTV-*c-myc* transgenic mice. Exogenous expression of the *c-myc* transgene, in addition to its ability to stimulate mammary gland neoplasia, has previously been shown to stimulate mammary gland hyperplastic and dysplastic changes (Amundadottir et al., 1995). Gross histopathological evidence, obtained from assessment of mammary gland whole-mounts, suggests that the *c-myc* transgene is active throughout the mammary epithelium of the tumor-bearing early parous and parous study mice.

Taken together, results from our study and from previous studies inform two possible and not mutually exclusive explanations: (1) breeding MMTV-*c-myc* mice at 7 weeks of age, as compared to 10 weeks of age, positively modulates the expression of the *c-myc* transgene; and (2) the mammary gland of the 7-week-old mouse is more susceptible to oncogene-mediated transformation than that of the 10-week-old mouse. Integration of viral DNA and transgenic constructs into the genome of mammals is often asso-

ciated with methylation and transcriptional inactivity (Doerfler et al., 1997). Methylation of the MMTV-LTR, silencing of linked transgene expression (*N-ras*<sup>N</sup> and *c-neu*) and abrogation of transgene-dependent tumorigenesis have been described previously (Mangues et al., 1995; Zhou et al., 2001). DNA methylation patterns are known to be most labile during periods of cellular transition such as embryogenesis and organogenesis (Robertson, 2002). At 7 weeks of age, the mammary gland in mice is actively growing and possesses numerous mitotically active terminal end bud (TEB) structures that continue to propagate the developing epithelial tree until it reaches its full extent at 10 weeks of age (Hennighausen & Robinson, 1998). It is possible that the MMTV-LTR undergoes a methylation state transition during the period between 7 and 10 weeks of age concomitant with alterations in the differentiation state of the mammary epithelium. If this is the case, pregnancy hormone stimulated expression of the MMTV-*c-myc* transgene and *c-myc*-mediated mammary tumorigenesis may be facilitated by the relative lack of MMTV methylation in the actively growing mammary epithelial cells (MECs). These actively dividing MECs of the TEB, present in the 7-week-old murine mammary gland, may prove more sensitive to transformation by the MMTV-*c-myc* transgene than the more quiescent, differentiated cells of the 10-week-old gland. These rapidly dividing cells may have reduced or absent DNA damage checkpoint systems and may propagate genetic mutations induced by the exogenous expression of *c-myc*. This possibility would mirror studies that demonstrate the height of rodent mammary gland susceptibility to chemical carcinogenesis is found during the TEB phase of glandular development (Russo & Russo, 1996). Our findings provide investigators with a tool for evaluating the influence of MMTV-LTR-directed transgene expression on mammary gland tumorigenesis in the setting of mixed genetic backgrounds.

### Acknowledgements

M.H. Jamerson is an MD/PhD student financially supported by the US Army Breast Cancer Research Program Pre-Doctoral Fellowship DAMD17-97-1-7110 and the Georgetown University Institutional Tumor Biology Training grant (2T32CA09686). NIH grants 1R01AG1496 and 1R01CA72460 to R.B. Dickson also supported this work.

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## EXPLANT-CELL CULTURE OF PRIMARY MAMMARY TUMORS FROM *MMTV-c-Myc* TRANSGENIC MICE

XU FANG PEI, MARCIA S. NOBLE, MARIA ANTONIETTA DAVOLI, EDWARD ROSFJORD, MADDALENA T. TILLI, PRISCILLA A. FURTH, ROBERT RUSSELL, MICHAEL D. JOHNSON, AND ROBERT B. DICKSON<sup>1</sup>

Lombardi Comprehensive Cancer Center (LCCC), Department of Oncology, Georgetown University Medical Center, The Research Building, 3970 Reservoir Road, Washington, D.C. 20007

(Received 18 November 2003; accepted 5 December 2003)

### SUMMARY

We have established an explant-cell culture system for mammary gland tumors from *c-myc* oncogene-expressing transgenic mice and potentially other transgenic strains. By coating culture dish surfaces with fetal bovine serum and using culture media supplemented with low serum and growth factors, the mammary tumor specimens could be maintained in culture for over 3 mo. Throughout the culture period, the explants produced abundant outgrowths of epithelial cells. As the outgrowths of epithelial cells filled the dishes, the explants were serially transferred from one dish to another—a process that could be repeated at least six times, thus providing a continuous supply of primary tumor cells. This culture system provides a useful tool for studying the biology of mouse mammary gland tumors and possibly tumors from other organ sites.

**Key words:** epithelial culture; serial outgrowth; gelatin; serum; differentiation.

### INTRODUCTION

The transgenic mouse has emerged as an important model system for studying etiologic factors involved in the onset and progression of human breast cancer. Transgenic mice expressing breast cancer-related oncogenes and other cancer-related genes, including *c-myc*, *c-H-ras*, *c-neu*, *wnt-1*, *int-1*, *hgf/sf*, *igf-1*, *cyclin D1*, and *bcl-2*, either individually or in combination, develop mammary hyperplasias and carcinomas with characteristic morphologies, latencies, and metastatic frequencies (Stewart et al., 1984; Pattengale et al., 1989; Munn et al., 1995). Our group has focused on mechanistic studies of *c-Myc*-induced tumorigenesis in the *MMTV-c-myc* model (Jarmerson et al., 2004). For these studies, we have used histopathologic methods with primary tumors as well as cell biologic methods with explant cultures. For this work, we have also created, with significant difficulty, several *c-Myc*-expressing mammary tumor cell lines (Amundadottir et al., 1996; Weaver et al., 1999; Liao et al., 2000). From our limited success in this respect, it became clear that there was still a need for a good primary culture system for the in vitro propagation of transgenic mouse mammary tumors to promote their biologic study in vitro. To date, there are only a few reports of the characterization of transgenic mammary tumor-derived cells in primary culture. One of our previous studies examined the karyotypic abnormalities displayed by established cell lines from eight mammary gland tumors of *MMTV-c-myc* transgenic mice (Weaver et al., 1999). From this study and from our follow-up histopathology study of this tumor type (Liao et al., 2000), it appeared that a major problem with traditionally derived cell lines from such

systems is the possibility of selection bias for particular cell types from a heterogeneous tumor (Cailleau et al., 1978). Thus, these cell lines may represent only a subset of the cell types and characteristics of the original tumor. An easily applied primary culture explant system could help to overcome this problem by allowing the growth of a greater diversity of cell types.

Explant-cell culture systems have long been applied to a variety of human normal and tumor tissues (Lechner et al., 1982). For example, we have previously succeeded in explant-cell culture of human liver (Pei and Harris, 1985) and esophageal mucosa (Pei et al., 1988) by using low-serum and low-calcium 199 medium supplemented with growth factors. In one of these systems, the effects of the chemical carcinogen nitrosamine were studied on human esophageal mucosa epithelial cells (Pei et al., 1990). We have also analyzed chromosomal changes in the mucosal epithelium adjacent to esophageal cancer by explant-cell culture (Su et al., 1988). Recently, a new explant-cell culture system for human prostatic cancer tissues has been described (Zwergel et al., 1998). This system used polyethyleneterephthalate (PET) membranes as a growth surface, coating of the membranes with elements of the extracellular matrix, and cocultivation of explants with heterologous fibroblasts. Up to 10 serial transfer steps could be performed with the same tissue specimen to produce outgrowths of cell monolayers (Zwergel et al., 1998).

In this article, we describe an explant-cell culture system for mammary gland tumors from two types of *MMTV-c-myc* transgenic mice. Several different culture conditions were tested to determine the optimal medium and dish surface preparation technique. The same technique was then applied to mammary and salivary gland tumors derived from *MMTV-tTA/tet-op-Tag/tet-op-ER $\alpha$*  transgenic mice (Tilli et al., 2003) with equal success. The mammary and

<sup>1</sup>To whom correspondence should be addressed at E-mail: dicksonr@georgetown.edu

TABLE 1

CELL OUTGROWTH FROM DIFFERENT MAMMARY GLAND TUMORS FROM A *C-MYC* TRANSGENIC MOUSE AFTER 7 C<sup>a</sup>

Tumors	IMEM <sup>a</sup> (0.5%)	MCDB151
88CT1	40/48	0/21
88CT2	10/22	0/18
88CT3	23/24	12/16
88CT4	8/8 (4 fibroblasts)	0/10
88CT5	10/11	2/8

<sup>a</sup> These data describe the number of outgrowths per number of tumor explants cultured.

<sup>b</sup> Abbreviation: IMEM, Iskoves minimum essential medium.

salivary gland tumor specimens in this culture system could be maintained over a 3-mo period, and they could be serially transferred many times (at least six) to new dishes to continuously give rise to epithelial cell outgrowths. These cell outgrowths could be subcultured, some were frozen and thawed without loss of epithelial cell characteristics, and some became immortal.

#### MATERIALS AND METHODS

**Tumor tissue source.** *MMTV-c-myc* and *MMTV-tTA/tet-op-TAG/tet-op-ERα* transgenic mice, with a variable number of mammary and salivary gland tumors (one to five) of different sizes, were sacrificed as approved in our institutional animal protocol. A sample (0.5–1 cm<sup>3</sup>) of tumor tissue was dissected from each tumor and maintained at 4° C in improved Iskoves minimum essential medium (IMEM; GIBCO, Grand Island, NY) containing antibiotics (100 u/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml gentamicin) before processing for culture. The tumor specimens were washed with L-15 medium, containing antibiotics, cut into small pieces of about 2 mm<sup>3</sup>, and plated into 60-mm tissue culture dishes or six-well plates. The dishes, plates, and flasks were precoated either by a brief rinse with fetal bovine serum (FBS) or by incubation with a solution of 1% gelatin-phosphate-buffered saline (PBS) at 37° C for 30 min. Any tumor fragments not placed in culture were frozen after suspension in IMEM containing 20% FBS and 7.5% dimethyl sulfoxide (DMSO) at –80° C to preserve the tissue for subsequent experiments. *MMTV-c-myc/vegf* bitransgenic mice were used in exactly the same manner as *MMTV-c-myc* mice. The *MMTV-vegf* strain (in the same FVB background as *MMTV-c-myc*) was generated by Dr. Glenn Merlino (National Cancer Institute [NCI], National Institutes of Health [NIH]). Metastatic, bitransgenic *MMTV-c-myc/vegf* tumors are the subject of another study (in prep.).

**Culture conditions.** The culture media tested for *c-Myc*-expressing tumor explant-cell culture included IMEM, F12–Dulbecco modified Eagle medium (DMEM) (GIBCO/BRL), and MCDB151 (Biofluids Inc, Rockville, MD). All media were supplemented with FBS (0.5–2%), epidermal growth factor (5 ng/ml), insulin (5 µg/ml), hydrocortisone (1 µg/ml), estrogen (E2, 20 nM), and antibiotics (100 u/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml gentamicin, all supplements from GIBCO/BRL). Initially, only a small volume of medium was added to each dish (1–1.5 ml) to prevent the explants from floating above the bottom of the dish; media were changed daily. After the explants attached onto the dishes, more media were added to just submerge the explants. The media were refreshed every 2–3 d, and the dishes were kept in a humidified incubator with a 5% CO<sub>2</sub> atmosphere at 37° C.

**Cell subculture and explant transfer.** Cell outgrowths from explants were examined frequently with an inverted, phase-contrast microscope. Those explants with fibroblast outgrowth were discarded immediately (Table 1), whereas the explants with epithelial monolayer outgrowths were retained in culture for long periods (over 3 mo). When the epithelial cell outgrowths formed a large halo surrounding the explants, the explants were carefully removed and transferred into new dishes with sterile forceps. The cells that had grown from the explant were subcultured, by trypsinization, into new plates or T25 flasks. The explants could be serially transferred several times to continuously provide epithelial cells. Some of the explants were transferred onto coverslips to provide primary tumor cells for immunocytochemical studies.

TABLE 2

CELL OUTGROWTH FROM *C-MYC/VEGF* TRANSGENIC MOUSE MAMMARY TUMOR EXPLANTS IN DIFFERENT MEDIA FOR 10 D<sup>a,b</sup>

Tumors	IMEM (0.5%)	IMEM (2%)	F12–DMEM	MCDB151
MVT	10/10	11/11	7/8, 1 floating	2/5, 5 floating

<sup>a</sup> These data describe the number of outgrowths per number of tumor explants cultured.

<sup>b</sup> Abbreviations: IMEM, Iskoves minimum essential medium; DMEM, Dulbecco modified Eagle medium.

All new culture dishes including coverslips were coated with serum or gelatin.

**Cell ploidy assays.** The ploidy of the transgenic mouse mammary tumor cells was examined by flow cytometric deoxyribonucleic acid (DNA) analysis and by counting the number of chromosomes. Duplicate samples of 1 × 10<sup>6</sup> Myc47F primary, unpassaged tumor cells, Myc87T1 × 2R mammary tumor cells at passage 2, and Myc88CT3 mammary tumor cells at passage 10 were collected, suspended in 100 µl citrate–DMSO buffer and stored at –80° C until analysis. Mouse spleen cells were also isolated and stored in citrate–DMSO buffer for comparison, as a 2n control. Cell nuclei were prepared for flow cytometry according to the method of Vindelov et al. (1983). Spleen cells were added to one of the duplicated samples before staining, to verify the diploid peak. Flow cytometric analysis was performed with a Becton Dickinson FACSsort flow cytometer.

For the preparation of chromosomes, several different isolates of mammary tumor cells were treated with 0.5 µg/ml colchicine (GIBCO) for 4 h. Cells were collected by trypsinization and placed in a 15-ml conical polystyrene tube. After centrifugation at 1000 rpm for 5 min, the cell pellet was treated with a hypotonic solution of 0.075 M KCl at 37° C for 1 h. Cells were centrifuged to decant the supernatant. The cell pellets were fixed with methanol–glacial acetic acid (3:1) for 30 min. The fixation was repeated two more times. The fixed cell suspension was dropped onto precleaned microscope slides and air dried. Chromosomal G-banding was performed by treatment of the slides with trypsin and staining with Giemsa (Jamerson and Dickson, in press). The chromosome numbers of different cell strains were counted using an Olympus VAAOX-S microscope. At least 20 metaphase spreads were counted for each of the cell strains.

**Cell-type characterization.** In general, it was straightforward to distinguish fibroblasts and other cell types by their microscopic morphologies. However, for more definitive characterization of the cell outgrowth from the mammary gland tumors, some explants were cultured on coverslips and stained for cytokeratins. When the cell outgrowths formed large halos of cells, the explants were transferred into new dishes. The cells left on coverslips were fixed in methanol for 20 min and permeabilized with 0.5% Triton X-100–PBS for 30 min. Immunostaining was performed for cytokeratin staining and to determine the expression of *c-Myc*. The primary antibodies used were broadly reactive to all cytokeratin types (Z0622, DAKO) and *Myc* (c-19, Santa Cruz Biotechnology, Santa Cruz, CA). Hematoxylin was used to counterstain. For more definitive characterization of the cell outgrowths from the salivary gland tumors, expression of the epithelial cell-specific (Hennighausen et al., 1995), tetracycline-regulated *ERα* transgene was measured by reverse transcriptase–polymerase chain reaction (Hruska et al., 2002) on whole ribonucleic acid extracted from explant-cell culture cells treated with phenol red–free IMEM media with charcoal-stripped serum in both the absence and the presence of tetracycline (1 µg/µl) for 72 h to downregulate *ERα* transgene expression in the cells.

#### RESULTS

Fourteen mammary gland tumors from six *c-myc* transgenic mice and one tumor from a *c-myc/vegf* transgenic mouse were subjected to explant-cell culture. In all cases, most of the attached explants developed cell outgrowths in 5–10 d (Fig. 1; Tables 1 and 2). There was substantial variation in the cell types that grew out from different tumors, both from the same and from different mice. Different types of cell were also observed from the same tumor and even from

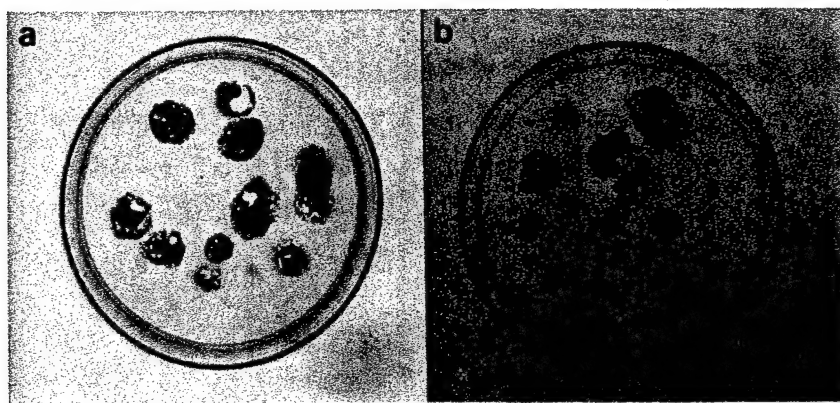


FIG. 1. (a) Cells grew out from the periphery of tumor explants and formed epithelial halos after 1 wk. (b) The tumor explants had been transferred three times with similar primary tumor cell outgrowth as before.

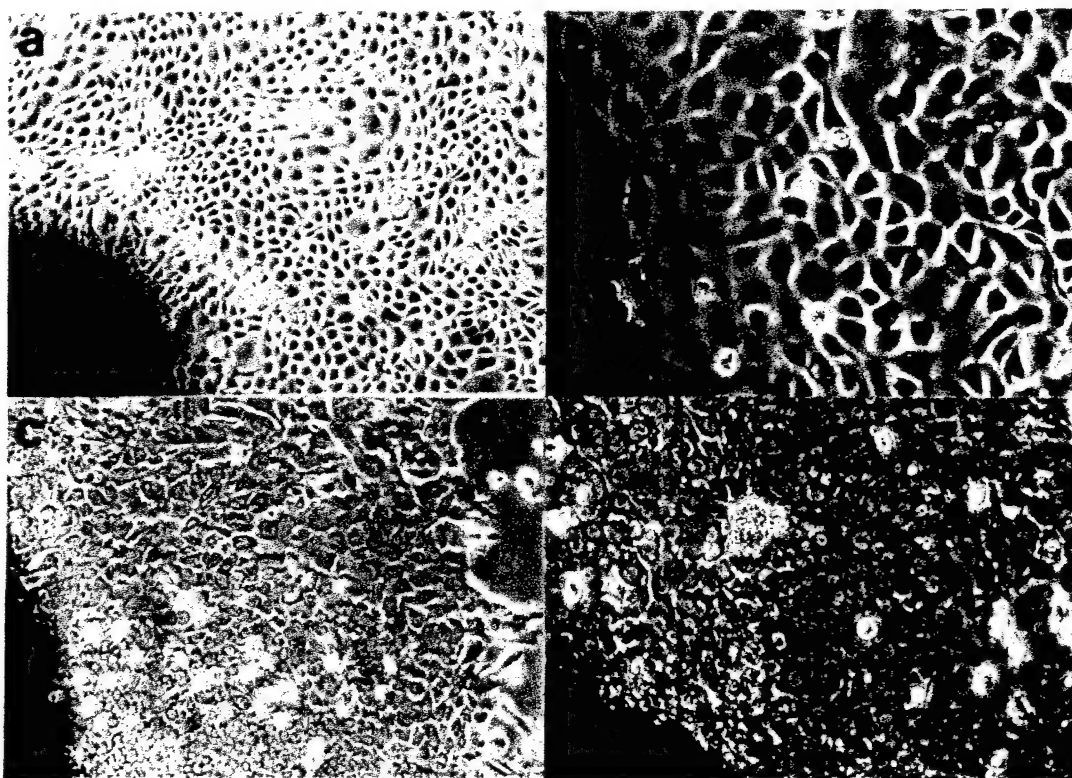


FIG. 2. Morphologically different cell outgrowths from explant-cell-cultured tumor tissues of Myc83C mouse mammary gland tumor 1 (83CT1: a,  $\times 10$ ; b,  $\times 20$ ) and tumor 3 (83CT3: c,  $\times 10$ ; d,  $\times 20$ ) on day 6.

the same explant. These different cell types varied in their capacity for in vitro growth (Figs. 2 and 3; Table 3). Most cells died at the first passage. However, many tumor cell strains were able to overcome a growth crisis and were passaged at clonal density for over 10 passages, with no sign of senescence. The explant-cell culture results are summarized in Table 3.

*Variations in cell outgrowth capacity among different tumors.* Although cancer cells are generally thought to be immortal, only a small portion of the cells that grew out from the tumor explants were able to grow indefinitely in vitro. It is not clear what factors determine their capability for in vitro growth. For example, three

tumors were removed from one mouse (Myc83C) and were used to generate a number of explant cultures. All the explants derived from tumor 1 (83CT1) had typical polygonal epithelial cell outgrowth morphologies (Fig. 2a and b), whereas the explants from tumor 3 (83CT3) produced mostly small round epithelial cells (Fig. 2c and d). The cells growing from 83CT1 and T3 died at their first passage. In contrast, most pieces of the second tumor (83CT2) gave rise to long, spindle-shaped cells (which were keratin positive, data not shown). These spindle-like cells were actively proliferating; numerous mitotic figures and rapid migration were observed (Fig. 3a and b). Some pieces of T2 grew out as small, round cells, but at the



FIG. 3. The Myc83C tumor 2 (83CT2) in explant-cell culture produced outgrowths with active proliferating cells (a,  $\times 10$ ; b,  $\times 20$ ). Note that at the edge of the halo, the small round cells either changed into spindle-like cells or were invaded by spindle-like cells (c,  $\times 10$ ). These cells have been cultured for over 4 mo and passaged 13 times (d,  $\times 10$ ).

TABLE 3

SUMMARY OF EXPLANT-CELL CULTURE RESULTS OF MAMMARY TUMORS FROM *C-MYC* TRANSGENIC MICE<sup>a</sup>

Mouse no. (date of culture)	Cultured tumors	Cell outgrowth <sup>b</sup>	Cell lines (passage number)
83C (April 4, 2000)	3	T1, T2, T3	83CT2 (p20)
5B (April 4, 2000)	1	T1	
87 (May 2, 2000)	2	T1 (T2 fibroblasts)	87T1a and 87T1b (p5)
85C (May 12, 2000)	2	T1, T2	
88C (June 6, 2000)	5	T1, T5, T3, T4, T2 (less)	88CT1 (p15) 88CT5 (p6) 88CT3 (p5)
6D (June 28, 2000)	1	T1	6DT1 (p18)

<sup>a</sup> These data describe the mouse from which the tumor was taken, the number of tumors from the mouse that were cultured, the nomenclature and consequently the number of outgrowths from the tumors, and the cell lines that were derived from the outgrowths.

<sup>b</sup> The values in parentheses indicate cell outgrowth morphologies other than epithelial. All other cell outgrowths were epithelial in appearance.

edge of the cell halo, they either changed into spindle-like cells or were invaded by spindle-like cells (Fig. 3c). These cells have a great growth potential in vitro and have been continuously passaged at low cell density ( $1: > 40$ ) every 4–5 d (Fig. 3d). To date, 83CT2 cells have been passaged over 20 times.

*The effects of culture conditions on cell outgrowth.* The capability of cancer cells for in vitro growth may be related to their malignant state or to their specific genetic alterations. However, in addition, environmental conditions (culture methods, media, different types of cell interaction, etc.) play a role by facilitating or inhibiting in vitro growth of cancer cells.

In the choice of culture media, because there was no well-established, general methodology for explant-cell culture of primary mammary gland tumors, we tested several culture conditions to establish a suitable culture system. We first chose three culture media with different concentrations of  $\text{CaCl}_2$ . IMEM (200 mM  $\text{CaCl}_2$ ) and F12-DMEM (116.50 mM  $\text{CaCl}_2$ ) are commonly used for culture of normal and malignant human and mouse mammary gland cells, whereas MCDB151 (3.32 mM  $\text{CaCl}_2$ ) was designed for serum-free, clonal growth of normal human bronchial epithelial cells (Lechner et al., 1981). Some laboratories have had success in culturing normal human breast epithelial cells and breast cancer cells with



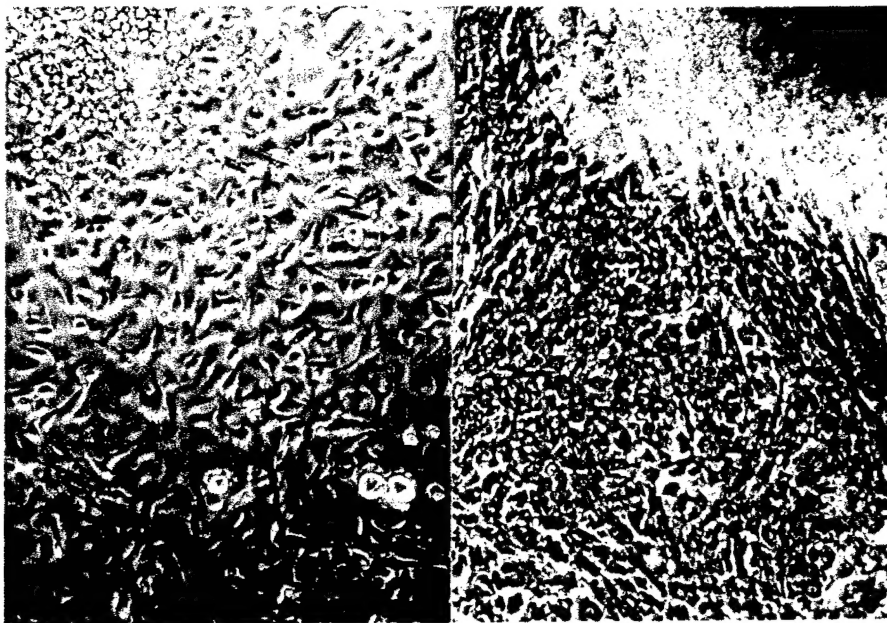


FIG. 4. A mammary gland tumor from a bitransgenic *c-myc/vegf* mouse in explant-cell culture produced two morphologically distinctive cell types (a and b).

MCDB170 (Ethier et al., 1993; McCallum and Lowther, 1996). However, our results indicate that complete IMEM was superior to the other two media in the context of our mouse mammary tumor explant culture. Cultured mammary tumor explants in complete IMEM gave rise to different types of cell outgrowths. Some of the cells could be passaged for long periods, and they appeared to be immortal in vitro. Healthy monolayer outgrowth was also obtained from explants cultured in complete F12-DMEM medium. However, it was difficult to passage the primary cell outgrowths in this medium. In MCDB151 medium, the attachment of explants to the surfaces of culture dishes was poor. Although MCDB151 was effective in inhibiting fibroblast growth, the migration of tumor cells from explants was also observed. However, explants cultured in MCDB151 medium did generate outgrowths that consisted of relatively pure cell types. Most cells grown in MCDB151 demonstrated a typical epithelial morphology. MCDB151 medium might be suitable for growth of cells from less malignant or well-differentiated tumors. In one experiment, we initiated explant cultures from samples derived using a tumor from a mammary gland of a *c-myc/vegf* bitransgenic mouse. Similar results were obtained with those from single transgenic, *c-myc*-derived tumors (Tables 1 and 2). Morphologically different cell types grew out from the explants of the *c-myc/vegf* transgenic mouse mammary tumor, and many explants also contained abundant fibroblasts; the latter observation differed from that of tumor tissues derived from *c-myc* transgenic mouse mammary glands (Fig. 4). Cells derived from *c-myc/vegf* tumors that had an epithelial morphology were collected; they stained positive for cytokeratins (Fig. 5) and c-Myc (Fig. 6).

We tested the effect of different concentrations of FBS on cell outgrowths. Tumor explants cultured in IMEM, containing 0.5% and 2% of FBS, each produced well-growing epithelial cells. However, explant attachment was better in IMEM containing 2% FBS than 0.5% FBS. Because a low concentration of serum is unfavorable for

fibroblast growth, we used 0.5% FBS in most of our explant-cell cultures and cell cultures. We routinely observed that medium containing 2.5% or more of FBS obviously favored fibroblast overgrowth.

Regarding the coating solution, explant-cell culture of mammary tumor tissues in dishes coated with FBS or 1% gelatin gave similar results. Because coating the culture surface with FBS was easy and efficient for facilitating tumor explant attachment, we used FBS in most of our explant-cell cultures. We believe that the initially high serum concentration, resulting from the coating procedure, favored cell attachment and initial growth and that the rapid dilution of the serum, resulting from medium changes, suppressed fibroblast overgrowth.

**Explant serial transfer and cell subculture.** Typically, 5–10 d were required for the outgrowing epithelial cells to form a halo of about 5 mm<sup>2</sup> surrounding the explant. When this layer of cells reached the desired size, the explants were removed carefully and transferred into new dishes, and the cells were subcultured by trypsinization and seeding into new flasks. Generally, the tumor explants could be maintained in culture for over 3 mo and transferred several times to continuously provide new epithelial cells. Some explants, with monolayer cell outgrowth, were transferred onto coverslips for immunostaining. The epithelial nature of outgrowing cells from both *c-myc* tumor-derived explants and *c-myc/vegf* tumor-derived explants was confirmed by positive staining for keratin filaments (Fig. 5). The expression of c-Myc was also detected in these cells by immunostaining (Fig. 6a). Tetracycline-regulated expression of the ER $\alpha$  transgene was detected in the cells derived from the salivary tumors (Fig. 6b). The culture of most explants was terminated before their capacity was exhausted, to produce further cellular outgrowths. Therefore, it was not determined how long it would be possible to continue to obtain cells from a single explant using this method. Furthermore, we passaged the cells that had grown from several of

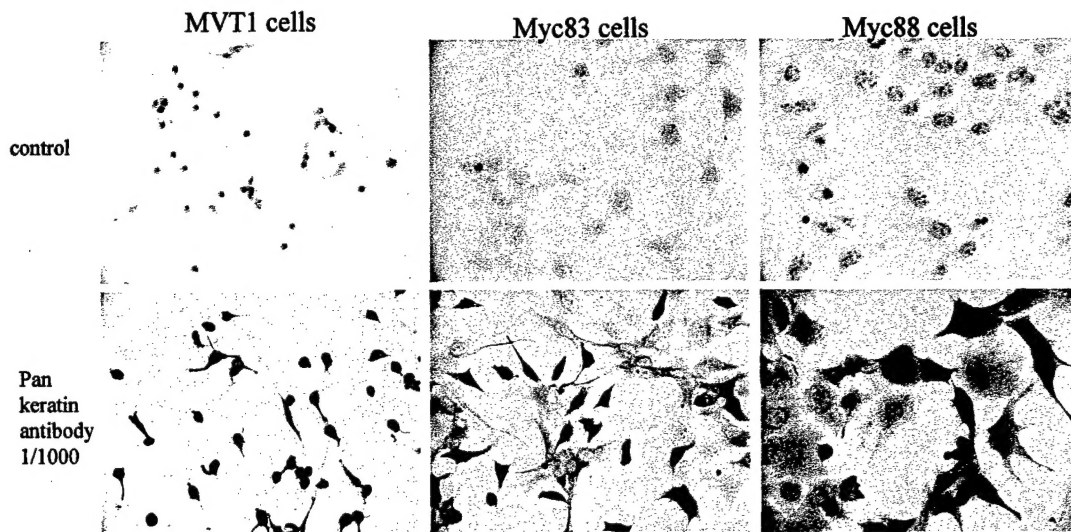


FIG. 5. Immunostaining of cytokeratin filaments. The top three panels from left to right are controls, excluding the primary, anticytokeratin antibody. Cells were derived from *c-myc/vegf* tumor explants (MVT), Myc83 cell line, and the *c-myc* tumor explants (88CT1), respectively. The bottom panels show positive immunostaining for epithelial-specific cytokeratins in all three cell types.

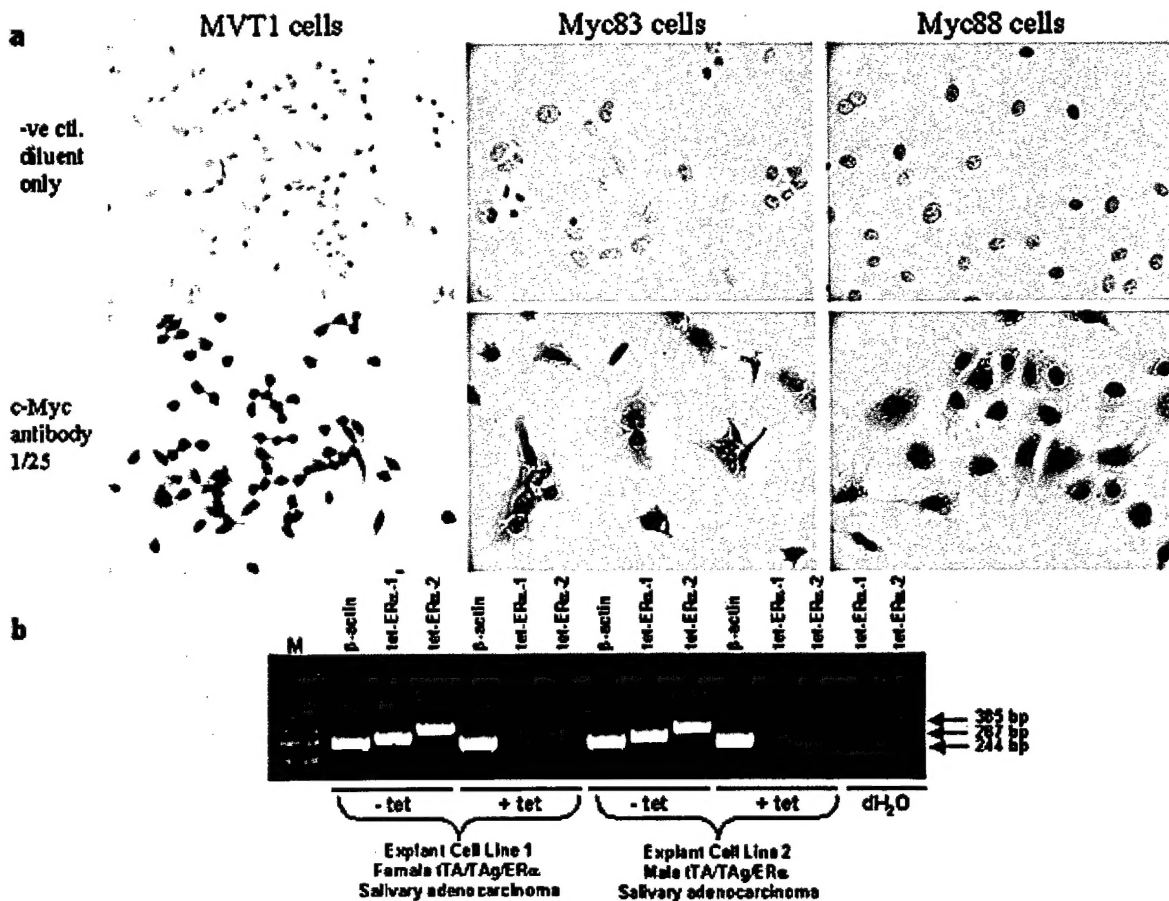


FIG. 6. (a) Immunostaining of c-Myc. The top three panels from left to right are controls, excluding the primary, anti-c-Myc antibody. Cells were derived from *myc/vegf* tumor explants (MVT), Myc83 cell line, and the *c-myc* tumor explants (88CT1), respectively. The bottom panels show positive immunostaining for c-Myc. The Myc83 cell line served as a positive control for c-Myc expression. (b) Cells from explant-cell culture of tTA/TA/ERα salivary adenocarcinomas demonstrate downregulation of ERα messenger ribonucleic acid (RNA) message (tet-ERα-1: 287 bp, tet-ERα-2: 385 bp) by reverse transcriptase-polymerase chain reaction (RT-PCR) after tetracycline treatment (+tet). β-Actin RT-PCR (244 bp) was performed as a control for RNA integrity and complementary deoxyribonucleic acid synthesis.



TABLE 4

CHROMOSOME ANALYSIS OF MAMMARY TUMOR CELL STRAINS DERIVED FROM *C-MYC* AND *C-MYC/VEGF* TRANSGENIC MICE\*

Cell strains	Cell number count	Chromosome no. (range)	Chromosome no. (average)
c-Myc tumor lines			
83CT2	32	38-78	41-42
6DT1	35	38-82	41-42
87T1b	24	40-90	72
88CT1	23	64-70	64
88CT1 epithelial	28	40-100	65
88CT3	24	70-78	72
c-Myc/Vegf tumor line			
MVT1	21	39-45	42

\*Tumor-derived cell lines were characterized for average chromosome number and range of number of chromosomes observed per cell.

the explants to generate cell lines, some of which appear to be immortal (Table 3).

**Cell ploidy.** The ploidy of the transgenic mouse mammary tumor cells was initially examined by flow cytometric DNA analysis. Both the primary mammary tumor cells that outgrew from the Myc47F tumor explants and the early-passage 87T1  $\times$  2R mammary tumor cells consisted of mixed diploid and triploid populations. In contrast, the later-passage 88CT3 tumor cells were predominantly triploid. These data agree with those from the chromosomal counting experiments. Frequent changes in chromosome number were observed in all cell strains, with most of the cells gaining an extra one or two chromosomes. These data are summarized in Table 4.

## DISCUSSION

Many attempts have been made to generate tumor cell lines for study of the biology and genetic changes involved in tumor initiation and progression. However, only a few human breast cancer cell lines have been derived from primary breast cancer tissues. Most human lines have been derived from tumor metastases or malignant pleural effusions (Butel et al., 1977; Cailleau et al., 1978; Band et al., 1990; Lewko et al., 1990; Calaf et al., 1992; Ethier et al., 1993). In reports of several hundred cases of human breast cancer primary culture, only 0.7-10% of tumors were able to grow in vitro and give rise to immortal cell lines (Cailleau et al., 1978; Amadori et al., 1993; McCallum and Lowther, 1996). Previous studies with mouse mammary tumors are consistent with this notion of a highly mixed population of tumor cells requiring enrichment of a tumorigenic subpopulation for xenograft growth. Recent research has demonstrated that only a small fraction of cells within these tumors are capable of initiating new tumors in immunocompromised mice. These tumorigenic cells appear to have a defined cell surface marker phenotype, allowing their isolation (Al-Hajj et al., 2003). Mouse mammary tumor lines generally have been derived from mammary tumors that had been serially passaged in vivo by transplantation into syngeneic mice over an extended period; only a few lines have been generated directly from primary mammary gland tumors (Kuzumaki et al., 1980; Band et al., 1990). Direct isolation of cells from tumors by collagenase allows the immediate production of large numbers of cells. However, most cancer cells produced in this

way are unable to attach and grow in primary culture. Those that do grow are often gradually overwhelmed by fibroblast overgrowth.

The explant-cell culture system described here provides the unique advantage of ease of monitoring cell outgrowth, facilitating the identification of portions of the tumor that are giving rise to fibroblastic outgrowths. These explants can be discarded immediately. The explants giving rise to epithelial outgrowths can then be serially transferred to new dishes to continuously provide epithelial cells. Alternatively, they can be transferred onto coverslips for biochemical studies. Most importantly, the cultured tumor tissues maintain a relatively physiological microenvironment, with the possibility for complex cell interactions found in the tissue. These interactions may support the long-term, in vitro survival of tumor cells of different types, which then can be studied as they grow out of the explants. Comparative analysis of these different cell types may allow better understanding of the inter- and intratumoral heterogeneity of mammary cancers. In addition, when the cell lines are grown out from tumors in which transgene expression is regulated by the tetracycline-responsive gene expression, tight regulation of gene expression is preserved in the derived cell lines.

Another explant-cell culture system for human prostatic cancer tissues used PET membranes, which were coated with extracellular matrix as a growth surface. In addition, the explants were cocultured with fibroblasts. Up to 10 transfers were performed to allow monolayer cell outgrowths from the same tissue specimen (Zwergel et al., 1998). However, this system allowed only short-term prostatic cancer cell cultures. Our explant-cell culture system for primary mammary tumors is simpler and easier to perform. The mammary tumor explants not only provide well-growing primary mammary monolayer tumor cells but also generate immortal cell lines of different cell types.

## ACKNOWLEDGMENTS

This work was supported by NIH RO1 CA72460 to R. B. D. and NIH RO1 CA89041 to P. A. F. We thank the LCC Animal Research, Tissue Culture, and Flow Cytometry Shared Resources (supported by U.S. Public Health Service Grant 2P30-CA-51008) for assisting this study. We also thank Dr. Glenn Merlino (NCI, NIH) for providing *MMTV-vegf* transgenic mice for this study.

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